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Comparison of Cytopathic Effects of Infectious Pancreatic Necrosis Virus Derived from Persistently Infected Cell Line and Carrier Fish

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Abstract. One adult black carp, *Mylopharyngodon piceus* (Richardson), with hemorrhage and ulcers on skin, fins and liver was used for the preparation of primary fish cell cultures. Only the swim bladder tissue was successfully cultured and the cells were designated SBC (swim bladder cell). SBC exhibited characteristics of transformed cell including loss of contact inhibition, anchorage independence and low serum requirement. A virus was isolated from the culture medium of SB cells but not from internal organs of the black carp. The agent was designated SB virus and was identified as infectious pancreatic necrosis virus (IPNV) (Chen, Kou & Chen 1993).

Cytopathic effects of SBC and internal organs of the carrier fish were compared using transmission electron microscope. The results showed that the cytopathic effects of these samples were the same including vacuolated endoplasmic reticulums, swollen mitochondrias and dilated perinculear cisternae. Cytoplasmic free virus particles and inclusion bodiew were seldom observed in the kidney but were frequently seen in the SBC. Viroplasms were somethimes observed in SBC.

INTRODUCTION

Black carp is a popular fresh water fish in mainland China and has gradually gained favor in Taiwan. This report describes a black carp with hemorrhage and ulcer on the skin and fins and was carrying IPNV.

IPNV, a member of the Birnaviridae, is an important pathogen in aquaculture, especially salmon and trout. In Taiwan, IPNV was isolated from several species of fish and clams (*Mertrix lusoria*) since 1980 (Chen, Chi, Guu, Chen & Kou 1984; Hedrick, Fryer, Chen & Kou 1983; Lo, Hong, Haung & Wang 1988), but no clinical signs were observed in most cases. This revealed the prevalence and importance of carrier fishes.

Carriers spreading IPNV by vertical and horizontal transmission is an important problem and makes prevention of this disease difficult. The prevention of persistent infection of fish is important. In this report, we described an IPNN persistently infected cell line which was derived directly from a carrier fish. These cells were different from other IPNV persistently infected cell lines (Ahne 1977; Hedrick, Leong and Fryer 1978; Hedrick & Fryer 1981; 1982; MacDonald & Kennedy 1979; Lo, Lin, Liu, Wang & Kou 1990). These cells were not established, in .vitro, from survivor cells of IPNV inoculated cell cultures but were established from carrier fish, and may provide a more mimetic model for the study of carrier fish.

MATERIALS AND METHODS

The adult black carp was collected in northern Taiwan. Primary cell cultures from liver, kidney, spleen, overy and swim bladder were prepared as described by Chen, Chi, Ueno & Kou (1983).

Both histological and electron microscopy examination were made from internal organs. The cultured cells of SBC were scrapped from the flask and centrifuged (2000rpm). Pellet of cultured SBC were also observed using electron micropsope. For light microscope, tissue fragments were fixed with Davidson's fixative and embedded in wax. Thick sections were stained with H & E stain and Giemsa stain. For electron microscope, samples were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer and were postfixed in 1% OsO_4 in 0.1M phosphate buffer, dehydrated in a series of ascending grades of ethanol, then substituted with acetone and embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate then examined with a Hitachi H-600 transmission electron microscope.

Virus isolation of this fish was performed two month after by inoculation of extract of internal organs (after freezed and thawed, internal organs in difficult to distinguish, so we mixed all internal organs together) onto monolayers of color carp tests (CCT) (Ku & Chen 1992) and TO-2 (Chen et al. 1983) cell cultures. The cell cultures were incubated at 18 ℃ and observed daily for the development of cytopathogenic effect (CPE).

Virus isolation from primary cell culture were performed by inoculating the culture medium of the primary cells into TO-2 and CCT cell cultures as described. The cell debris was pelleted (3000 rpm for 30 min) after complete CPE was observed, then separated on 10% polyacrylamide gel and identified by western immunoblot using rabbit anti-IPNV AB serotype polyclonal antibody (Chi and Chen 1991).

RESULTS

Only the primary culture of swim bladder was not contaminated by bacteria.

Swim bladder cells (SBC) migrated out from tissue fragments, propagated quickly and exhibited characters of transformed cell including loss of contact inhibition, anchorage independence (could be cultrued in suspension), and low serum requirement (decreased to 3% foetal bovine serum by the fourth passage) (Fig.1). Cytopathic effect including cellular degeneration and lysis were observed two days after the addition of culture medium from primary cells into CCT and TO-2 cell cultures. The agent isolated from SB cell was referred to as SB virus.

No CPE was observed in TO-2 and CCT cell cultures inoculated with diluted tissue extract and no further repassage of the inoculum was done.

For light microscope, rod shapped bacteria were observed in the liver. Vacuolated degenerative and necrotic cells were occassionally present in the liver and kidney. No parasites were observed.

Tissues of kidney, liver and spleen were examined using the transmission electron microscope. In the kidney, depth of nuclear furrow and unidentified inclusions within the furrow were occassionally observed (Fig.2). The cytoplasm of some interstitial cells and granulocytes were vacuolated. The perinuclear cisternae (nuclear envelope) of some nuclei appeared distented, and granules and vesicles were present in these spaces (Fig.3). Virus particles were difficult to differentiate from densely stained cytoplasmin granules. In many cases, virus particles were observed bound to membranes. Icosahedral virus particles and viral nucleocapsids were rearely found in the cytoplasm of kidney cells (Fig.4). Diameter of these virus particles was about 55-65 nm. The principle morphological properties of liver cells were well developed rough surfaced endoplasmic reticulums and mitochondrias, but in many cells the mitochondrias and endoplasmic reticulums had lyzed (Fig.5).

Cultured SBC wre observed by electron microscope. The cytoplasm of many SBC was highly vacuolated (Fig.6), harbouring swollen densely stained mitochondrias and vacuolated endoplasmic reticulums (Fig.6a & Fig.7). In many cases, the cytoplasm of these cellls showed randomly distributed free virus particles (Fig.6b), viral inclusion bodies and viroplasms (Fig.8 & Fig.9). Some of the vacuolated cells exhibited severely distended perinuclear cisternae enclosing pleomorphic vesicles and particles in this space (Fig.10). In completely lyzed cells, numerous free virus particles, nucleocapsids and tubular viral nucleocapsids were presented (Fig.11). The diameter of these unenveloped hexagonal virus particles which were found in the kindey (Fig.4).

The result of western immunoblot revealed that this virus was infectious pancreatic necrosis virus, but did not belonging to the AB, Sp or VR299 serotype. The molecular weight of VP2 polypeptide of this virus was about 50 KD (Fig.12 arrow) was larger than AB serotype but smaller than Sp serotype. From another immunological experiments using AB serotype specific anti-VP3 monoclonal antibody (Chi & Chen, 1991), the SB virus was identified as a variant of IPNV AB serotype (date not shown).

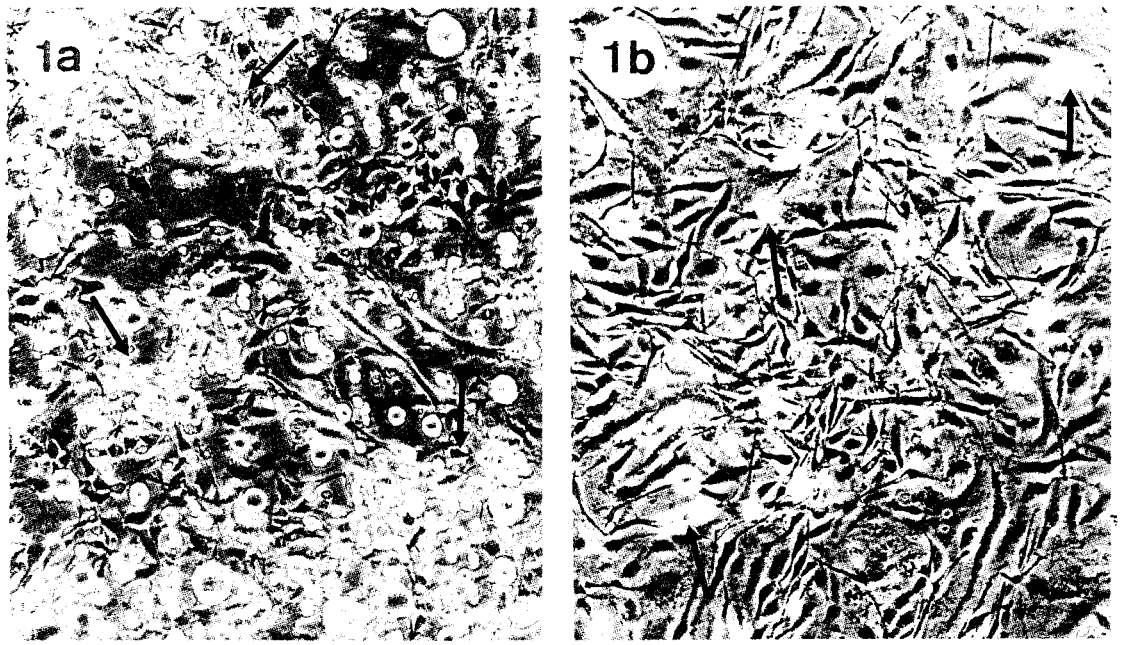

Fig. 1a Phase contrast micrograph of primary SB cells. Primary SB cells migrated out of tissue fragments, propagated and began to pile up (arrow), then peel off the cell monolayer and become a suspension culture. x370.

Fig. 1b Loose monolayer of SB cells were spindle shaped. Many mitotic figures were observed (arrow). x370.

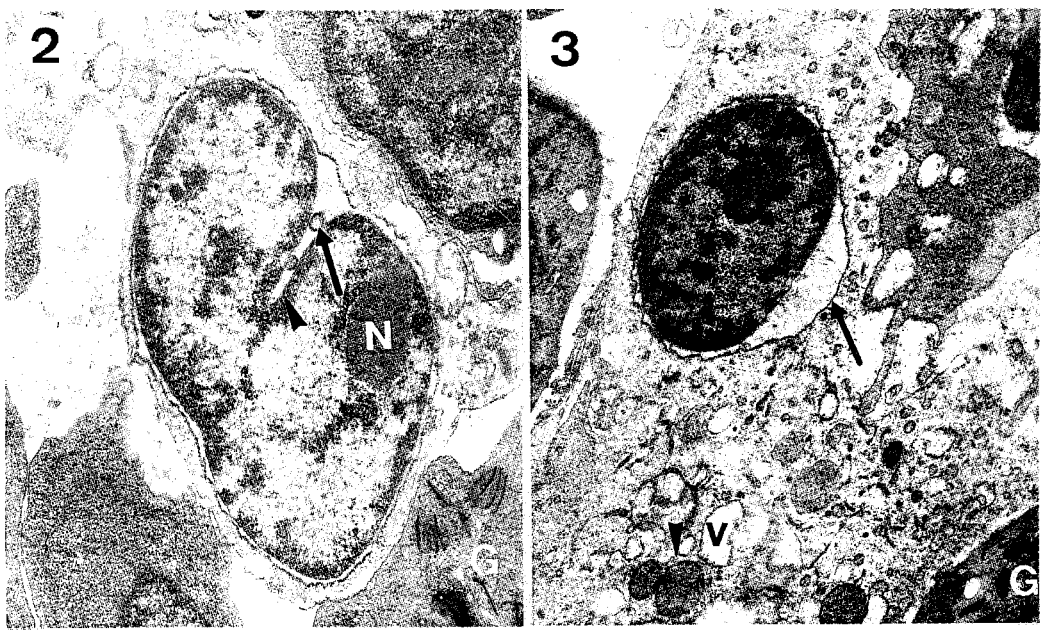

Fig. 2 Electron micrograph of kidney cells. This cell showed nuclear furrow (arrow head) and two unrecognizable inclusions (arrow) within nuclear furrow. N: nucleolus; G: portion of granulocyte. x17,000.

Fig. 3 Nucleus with distended nuclear cisternae (arrow) was observed occasionally in the kidney. Arrow head: swollen denselly stained mitochondria; v: vacuoles (were the dilated endoplasmic reticulum); G:Portion of granulocyte. x15,000.

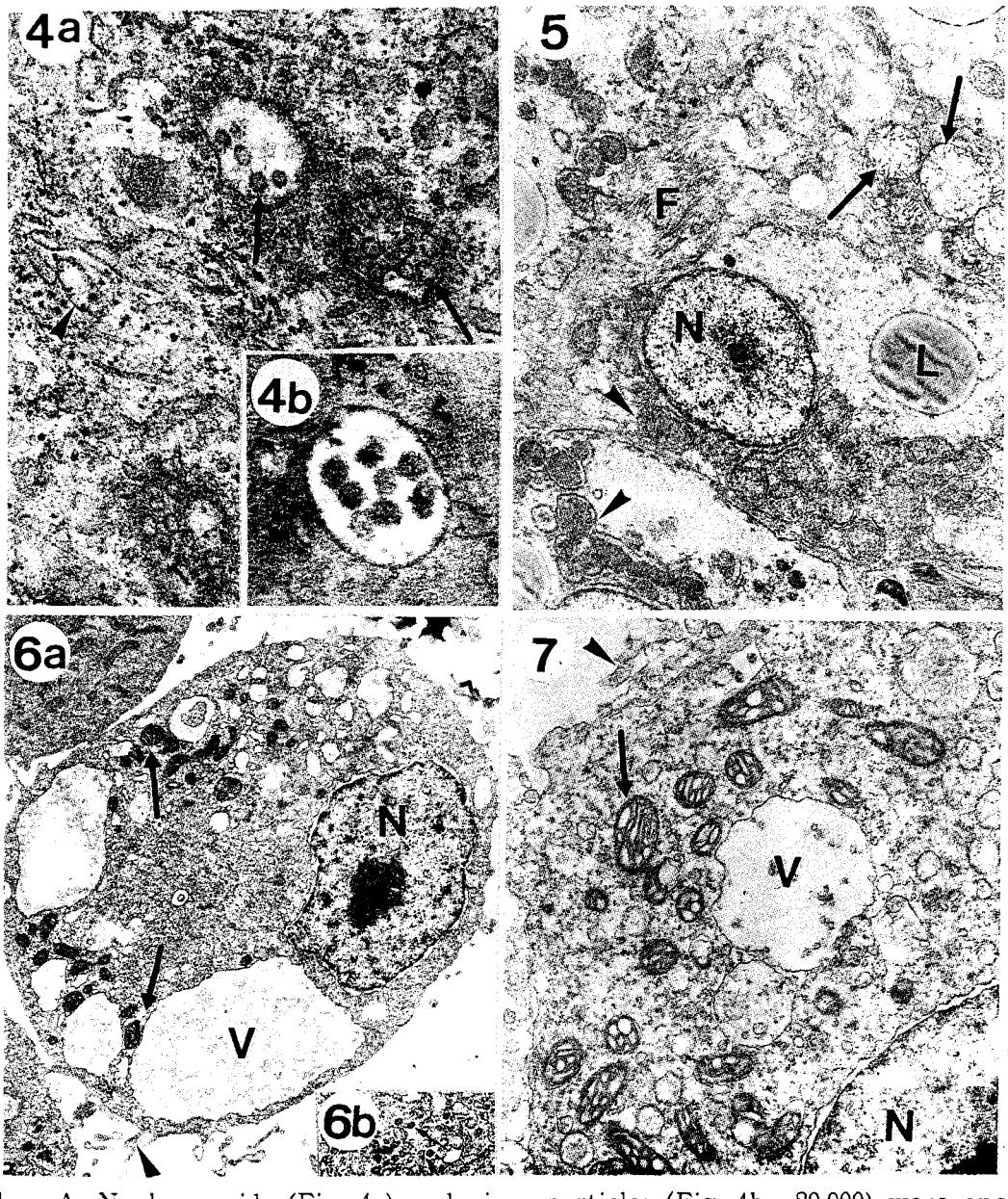

Fig. 4 A: Nucleocapsids (Fig. 4a) and virus particles (Fig. 4b x80,000) were enclosed in vacuoles (arrow) presented in the cytoplasm. Diameter of these virus particles were about 55-65 nm. Arrow head: dilated endoplasmic reticulum. x50,000.

Fig. 5 In the liver, many regions of necrotic mitochondrias (arrow) and mitochondrias surrounded by endoplasmic reticulum (arrow head) were observed. N: nucleus; L: lipid; F: fibril. x6,000.

Fig. 6 Electron micrograph of SB cell. Swollen densely stained mitochondrias (arrow), vacuolated endoplasmic reticulums (v), viral nucleocapsids (small black arrow in Fig. 6b) and free virus particles (small black arrow) (Fig. 6b x17,000) were distributed randomly in the cytoplasm. N: nucleus; arrow head: microvilli of SB cell. x5,000.

Fig. 7 Swollen mitochondrias (arrow) and vacuolated endoplasmic reticulum (v) of SB cell. Most SB cell had numerous microvilli (arrow head). N: nucleus. x15,000.

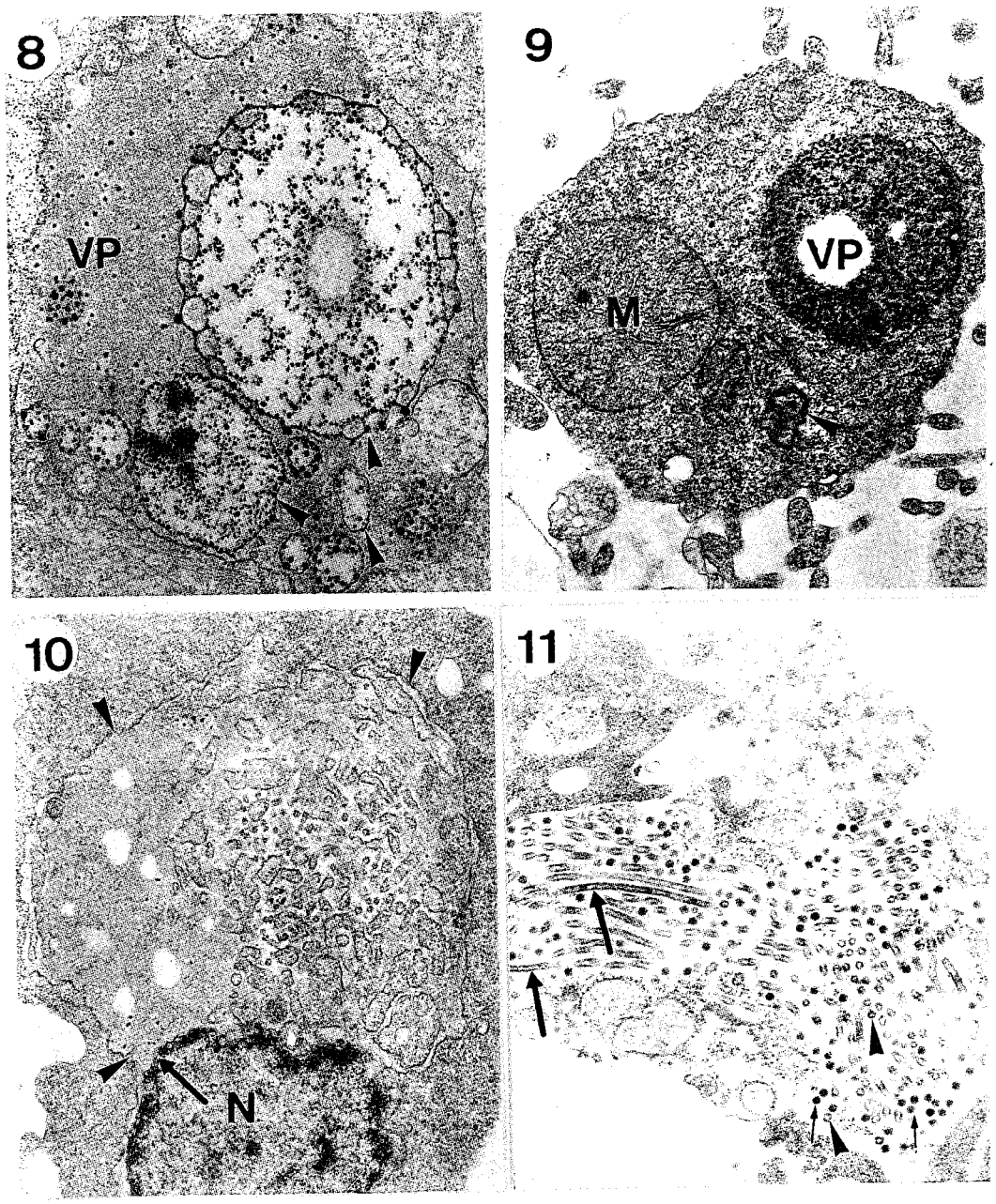

Fig 8. Inclusion bodies (arrow head) and viroplasm (vp) complexes were observed in the cytoplasm of SB cell sometimes. x.20,000.

Fig 9. Viroplasm (vp) with nucleocapsids, mature virus particles and amorphous material displayed in the cytoplasm of SB cell. Myelin-like membrane whols (arrow head) could be found in the cytoplasm also. x15,000.

Fig 10. Dilated perinuclear cisternae were frequently observed. In the dilated perinuclear space, some vesicles, unidentified inclusions and granules were often observed. Arrow: inner nuclear membrane; arrow head: outer nuclear membrane; N: nucleus. x15,000.

Fig 11. Tubular viral nucleocapsids (large arrow), nucleocapsids (arrow head) and virus particles (small arrow) accumulated in the lyzed cell. 225,000.

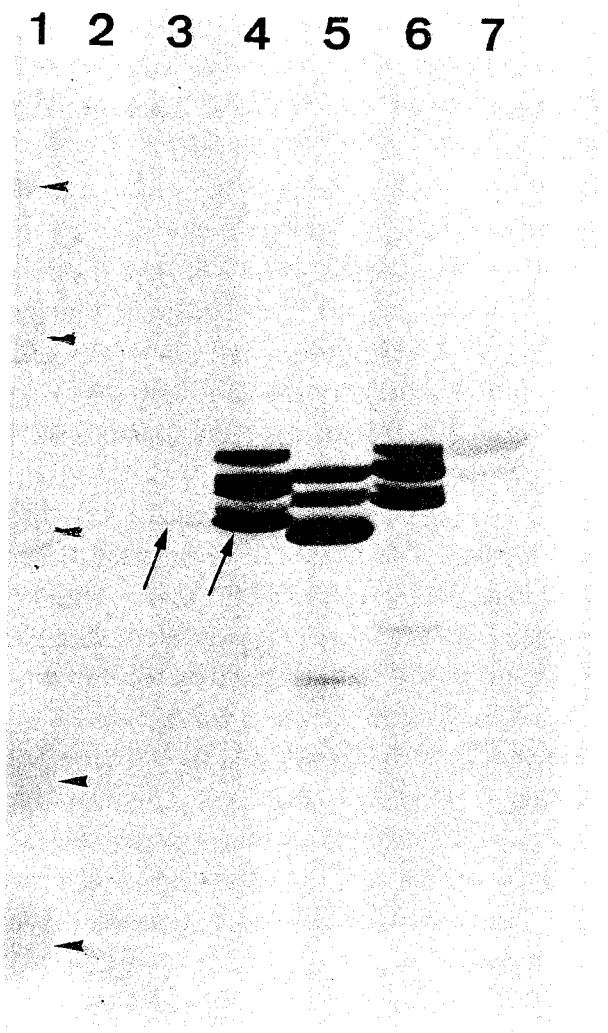

Fig 12. Comparison of SB virus cell associated antigens with IPNV AB, Sp and VR299 serotype by western immunoblot. Lane 1-7: Bio-Rad prestain protein molecular weight marker, the molecular weight of these five bands (arrow head) from top to bottom were 116.5, 80, 49.5, 32.5, 27.5 respectively, CCT cell debris, SB cell debris, SB virus antigens in CCT cells , IPNV AB serotype antigens in CCT cells, Sp serotype antigens in CCT cells and VR299 serotype antigens in CCT cells.

DISCUSSION

SB cell line is persistently infected with a virus. From the culture medium of SBC, SBV was isolated and had a titer about 10^6-10^8 $TCID_{50}$/ml. The persistent virus obtained from the SBC was indentified as IPNV.

When diluted tissue extract was inoculated onto CCT and TO-2 cell cultures, no CPE were observed. Because 1)in the adult IPNV carrier, only the posterior part of the kindey can be used effedtively for virus isolation (Yamamoto 1974; Mangunwiryo

& Agius 1988), but we disregarded this tissue. 2) The number of virus in the carrier was small, the CPE might develop slowly and require repeated passage. 3) Before virus isolation, the internal organs of this fish were freezed (-20℃) and thawed several times during storage which would cause inactivation of the virus. This may explain why we failed to isolated virus from tissues of this fish.

Histopathological effects in the carrier fish was not severe and were located mostly in the kidney. Pathology included vacuolated cytoplasm, swollen densely stained mitochondria, dilated endoplasmic reticulum and severely distended perinuclear cisternae containing granules. Virus particles present in the cytoplasm were not rigid in hexagonal profile but had a diameter about 55-65 nm and were associated with inclusion bodies of cell membrane. This feature was also observed by Lightner and Post (1969) in pancreatic aciner cells of rainbow trout, and by Yamamoto (1974) in kidney cells of brook trout.

Many cells in the SB cell line displayed cytopathic effects as described. Relatively large numbers of virus could be found in the cytoplasm, cytoplasmin inclusion bodies, viroplasms, and cell debris. Viroplasms were also occassionally observed in the cytoplasm, this observation has not been mentioned in the previous reports (Moss & Gravell 1968; Wolf & Quimby 1971). Viroplasm is either the normal mechanism of IPNV replication or is a special mechanism used by the SB virus replication in the SB cells. Distended perinuclear cisternae in not normally observed in RNA virus infected cells. This cytopathic effect implicates the integration of complementary DNA by the SB virus into the host's chromosome or coinfection of IPNV with a second virus containing DNA. In the previous reports, IPNV was isolated with/without coinfecting virus from tumorous tissues of eel and masu salmon (Ahne & Thomsen 1985; Yoshimizu, Homura, Awakura & Kimura 1988; Ueno, Chen, Kou, Hedrick & Fryer 1984). We are not able to prove a tumorous origin for the SB cell line but the transformation exhibited by the cells in primary culture suggest this cell line was derived from tumorous tissue.

Compare the cytopathic effect of the carrier fish with SBC, the SBC exhibit more severely than the carrier fish. The reason of this difference may be that the carrier fish could release and dilute the SBV into environment, but SBV was released and accumulated in the culture medium of SBC and superinfceted the SBC cultured in the same flask. Therefore, SBV was detected in the culture medium and cytoplasm of SBC easily.

The persistent virus obtained from SB cell was classified as a IPNV AB serotype but has a larger VP2 polypeptide than the reference AB serotype virus (Fig.12). In previous reports, the persistence of IPNV in the PI cell line has been attributed to the participation of detective interfering (DI) particles in the host cells (Hedrick & Fryer 1981; 1982; Kennedy & MacDonald 1982; Lo et al. 1990; 1991; MacDomald & Kennedy 1979; MacDonald & Yamamoto 1978), and the VP2 polypeptide of DI particles is smaller than the standard virus (Lo et al. 1991). Confer our results with these reports, we inferred that the persistence of SB virus couldn't be attributed to DI

particles. We prefer to assume that the persistence of SBV was caused by the mutation of VP2 polypeptide. The coevolution of SB virus and the host cells elicited the transformation of the SB cell and stabilized the persistence of SB virus in the SB cell.

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比較傳染性胰臟壞死病毒於持續性感染細胞株及帶原魚之細胞病變

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本細胞株取材自一尾表皮、鰭及肝臟有出血及化膿之成母青魚的膿組織。被命名爲SB細胞株。SB細胞株表現出失去接觸抑制性、失去停洎依賴性及血清依賴性降低等轉型細胞特性。自SB細胞培養液可分離出病毒，但自來源青魚則無法分離出。分離出來的病毒命名爲SB病毒且經鑑定爲傳染性胰臟壞死病毒（IPNV）。本論文利用穿透式電子顯微鏡比較SB細胞及來源青魚組織之細胞病變。結果顯示兩種來源之細胞病變相同，包括內質網空泡化、粒線體腫大及核膜擴張。在腎臟細胞內偶爾可見細胞質內病毒顆粒及病毒包涵體而在SB細胞內卻常可見。病毒質在SB細胞中有時可見。